

HOST DEFENCE RESPONSES IN PIGS EXPERIMENTALLY INFECTED WITH *SALMONELLA* TYPHIMURIUM.

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INTRODUCTION

Salmonella Typhimurium may induce persistent infections (organ carriers) in pigs, and healthy carriers seems to be a problem in controlling the infection (8). Evasion or disturbance of the host defence responses may be necessary for establishment of a persistent infection. Functional changes in circulating neutrophils from pigs infected with *Salmonella* Typhimurium have been demonstrated (1). The aim of the study was to investigate host defence mechanisms in pigs that have cleared the infection, persistently infected pigs and uninfected pigs, studies that may contribute to the explanation of how the carrier-state is established. Phagocytosis and oxidative burst in peripheral blood or cells isolated from peripheral blood were investigated in three inoculation experiments.

MATERIALS AND METHODS

Experimental *Salmonella* infection: Specific pathogen free (SPF) pigs, about 8 weeks old, were orally inoculated with *S. Typhimurium*. Before inoculation the pigs were allowed to acclimatize for about one week during which period the pigs did not excrete *Salmonella* in feces and were proven serologically negative to *Salmonella*. At slaughter, internal organs were investigated for *Salmonella* to assess the carrier state. During the experiments individual fecal samples and blood samples were taken with regular intervals.

Experiment 1: Seventeen pigs were inoculated with 10^8 cfu of *Salmonella* Typhimurium phagetype 12 and nine pigs were held as controls. Groups of pigs were slaughtered at day 16, 23-24 and 36-38 post inoculation. **Experiment 2:** Forty pigs were inoculated with 10^{10} cfu of *Salmonella* Typhimurium phagetype 12 and 20 pigs were held as controls. About 18 hours post inoculation groups of 10 pigs were treated once i.m. with a therapeutic dose of different antibiotics (ampicillin, enrofloxacin, dihydrostreptomycin) or saline, respectively. Pigs were slaughtered at day 70 - 80 post inoculation. **Experiment 3:** Eighteen pigs were inoculated with 4×10^9 cfu of *Salmonella* Typhimurium phagetype 12 and 9 pigs were held as controls. Groups of pigs were slaughtered at day 23, 44 and 58 post inoculation. Two inoculated and one control pig died during the first week of the experiment by causes that were not related to *Salmonella* infection. Data from these pigs are excluded.

Bacteria: A Rifampicin resistant strain of *Salmonella* Typhimurium phagetype 12, one of the common isolated strains from Danish slaughter pigs, was used in the inoculation experiments. The bacteria were grown at 37° C in nutrient broth over night without agitation, after which the bacteria were harvested and suspended in saline and the actual cfu/ml was measured.

Microbiology: Fecal samples were investigated for *Salmonella* to evaluate bacterial excretion. Verification of *Salmonella* was performed as follows: Samples of 5 g feces were inoculated in 45 ml nonselective pre-enrichment buffer and incubated at 37 °C over night. From the buffer, selective enrichment was performed in parallel with two methods: 1) 100 µl onto MSR/V agar plates and 2) 1 ml into 9 ml selenite-cystine broth. Both media were incubated at 41.5 °C for 18-20 h. *Salmonella* suspect colonies by MSR/V and from selenite-cysteine broth were subcultivated

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at Brilliant Green agar and isolation of the challenge strain was verified by detection of the O5-antigen and the rifampicin resistance. Tissues, 3 g of internal organs, were surface decontaminated by dipping in boiling water. The materials were homogenized in a Stomacher before pre-enrichment in 27 ml buffer. The microbiological analysis was performed as described for fecal samples.

Serology: Seroconversion was estimated by use of LPS- MIX - ELISA (4).

Cell-populations: Leucocytes in whole blood were counted (AUTOCOUNTER AC 900, SVELAB) and subpopulations estimated by flowcytometry using forward -and sidescatter or after incubation with Mabs against porcine CD4, CD8, CD2 and IL-2 receptor, respectively. The cell suspensions were analysed by flowcytometry a FACScan, Becton Dickinson, after fixation in 0.5 % paraformaldehyde.

Phagocytosis: Heparinized blood (100 ul) were incubated with heat killed FITC-labelled salmonella bacteria or zymosan A and Krebs Ringer balanced saline (KRBS) ad 1 ml for 40 min at 37° C under shaking conditions, after which the phagocytosis was stopped and the red blood cells lysed. After washing with PBS-EDTA (0.1 %) to remove noningested organisms the cells were resuspended in FACS Flow, fixed in 0.5% of paraformaldehyde and analysed by flowcytometry. FITC-labelling of salmonella bacteria and zymosan was performed by a method described by Gelfand et al. (2).

Oxidative burst was measured in peripheral phagocytic cells. Luminol-enhanced chemiluminescence (6) was after stimulation of isolated mononuclear leucocytes with phorbol myristate acetate (PMA), preopsonized zymosan A or buffer measured and the peak responses were used for estimation of oxidative burst capacity (experiment 1 and 2). Flowcytometric analysis of oxidative burst (5) was measured in whole blood after stimulation with PMA, FITC-labelled salmonella bacteria or buffer (experiment 3). After incubation for 40 min at 37° C under shaking conditions the red blood cells was lysed by addition of lysisbuffer. The cells were washed in PBS-EDTA (0.1%), resuspended in FACS Flow and fixed in 0.5% of paraformaldehyde. Flowcytometric analysis was performed and the relative fluorescence index was calculated as: % positive cells x mean fluorescence/100.

RESULTS

Clinical signs: Almost all pigs had subclinical infections without or with only slight temperature increase, and loose feces or slight diarrhoea for a few days after inoculation. In experiment 2, some pigs had a slight diarrhoea during the acclimatization period.

Microbiology: Most pigs excreted bacteria in feces for 4-5 weeks p. i., after which the excretion became intermittent. However, reinfections of pigs that have cleared the infection may have occurred in pigs from the same pen. In the group of pigs treated with enrofloxacin (experiment 2) the excretion was reduced. At slaughter *Salmonella* was demonstrated in internal organs in some infected pigs (Table 1), but never in controls.

Seroconversion: All inoculated pigs seroconverted about 7 -10 days p. i. and maximal levels of antibodies were measured after about 2 - 3 weeks, after which the levels declined. In some pigs the level of antibodies declined very slowly or was high (persistent) for more than 5 weeks. The level of seroconversion was reduced in pigs treated with enrofloxacin.

Leucocyte counts: WBC/ml blood was measured with regular intervals in experiment 3. The total number of leucocytes varied from 15 to 40 x 10⁶ cells/ml and was almost similar in inoculated and control pigs. The number of lymphocytes was reduced in inoculated pigs at day 2, 3 and 10 post inoculation as compared to control pigs, whereas for monocytes no significant differences were found. The number of granulocytes seemed to vary in two phases: An increase about 2-3 days p. i. followed by a reduction about 14 days p. i. after which values were

normalized at day 17-21 p. i. Subpopulations of lymphocytes were estimated by use of Mabs (CD 2, CD 4, CD 8). Large variations in percentage of positive cells during the experiment and between pigs were found but no obvious differences in percentage of subpopulations between inoculated and control pigs were found. IL-2 receptor positive lymphocytes (activated T-cells) were not found in peripheral blood samples during the experiment.

Table 1. Verification of *Salmonella* Typhimurium in internal organs.
Number of *Salmonella* positive pigs at slaughter after experimental infection.

Internal organs	Experiment 1 Day p. i.			Experiment 2 Day 70 - 80 p. i. (Group)				Experiment 3 Day p. i.		
	16	22-23	36-38	A	B	C	D	23	44	58
Tonsil	2/3	2/6	3/10	7/10	2/10	6/10	4/10	5/6	4/5	2/5
Mandibular	2/3	2/6	2/10							
Spleen	1/3	1/6	0/10							
Liver	0/3	2/6	0/10	2/10	0/10	4/10	2/10	3/6	1/5	0/5
Gall bladder	0/3	1/6	0/10							
Ileocaecal	3/3	4/6	2/10	2/10	0/10	2/10	1/10	6/6	1/5	0/5
Mesenterial	0/3	3/6	0/10	0/10	0/10	0/10	0/10	1/6	1/5	1/5
Colon lymphnodes	3/3	4/6	1/10	0/10	0/10	1/10	0/10	6/6	1/5	0/5
Intestine wall	3/3	3/6	2/10	0/10	0/10	2/10	0/10	5/6	3/5	0/5
Caecum content	1/3	5/6	4/10	5/10	2/10	4/10	3/10			
Feces	3/3	3/6	1/10							

Phagocytic capacity: The phagocytic activity against *Salmonella* in whole blood from inoculated pigs was enhanced compared to control pigs ($P=0.003$) at day 17 p. i., where maximal levels of antibodies were measured. The enhanced phagocytic activity was only found for granulocytes.

Oxidative burst: Oxidative burst, measured as luminol-enhanced chemiluminescence in monocytes after induction with PMA, was increased before inoculation and seemed to be normalized in control pigs after about 10 days of acclimatization. Large variation in the capacity of oxidative burst in cells when stimulated with PMA was measured both by chemiluminescence and by flowcytometry. Comparing inoculated and control pigs no obvious difference in the responses after stimulation with PMA was found but some pigs showed an increased response at 2-3 days p. i. (experiment 1 and 2). In inoculated pigs (experiment 2, no antibiotic treatment) an increased spontaneous response (buffer) was measured at day 14 post inoculation compared to control pigs. In experiment 1, high spontaneous response (buffer) was measured at day 3 p. i. in 3 of 9 inoculated pigs.

DISCUSSION

The capacity of host defence seems to be highly influenced by e. g. stress caused by environmental events. (3,7). This may have contributed to the large variance in cell responses between pigs in our experiments. Most of the differences in cellular responses between inoculated and control pigs were measured in the early phase of the infection, day 2 - 3, and about 10 to 14 days p. i. Biphasic responses were also found in pigs by Coe et. al. (1) after oral inoculation with

Salmonella Typhimurium. They found functional changes in circulating neutrophils with enhanced bactericidal activity against *Salmonella*, but depressed oxygen-dependent bactericidal activity (1). Our experiments indicated an increased oxidative burst in some pigs 2 - 3 days p. i. in PMA stimulated monocytes and an increased oxidative burst in unstimulated monocytes 14 days p. i. Induction of antibodies in inoculated pigs enhanced phagocytosis by granulocytes, but not in monocytes. This may indicate that granulocytes preferably recognize bacteria coated with antibodies, whereas opsonophagocytosis by monocytes seems more complement mediated.

In our experiments fifteen pigs were estimated as carriers (organ carriers) and a possible correlation between different cellular defence responses in pigs and the ability to clear the infection is further investigated.

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